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<b>(54) Title:</b> ATTACHMENT OF ACID MOIETY-CONTAINING BIOMOLECULES TO ACTIVATED POLYMERIC SURFACES  <b>(57) Abstract</b>  Compositions comprising an activated polymeric substrate having an acid moiety-containing biomolecule covalently bonded via the acid moiety to the substrate are disclosed. Methods for making such compositions are also provided, including methods that involve plasma processing of the substrate prior to the attachment of the biomolecule. These compositions find use as novel biomaterials, e.g. for tissue engineering and the prevention of adhesions, for selective attachment and growth of specific cell types, for coating surgically-implanted materials, and in coating vessels for storage of blood and blood products.		

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## ATTACHMENT OF ACID MOIETY-CONTAINING BIOMOLECULES TO ACTIVATED POLYMERIC SURFACES

### FIELD OF THE INVENTION

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This invention relates to compositions and methods for attaching certain biomolecules to polymeric surfaces. More particularly, the invention relates to polymeric substrates having acid moiety-containing biomolecules attached to the surface thereof and methods for making such modified substrates by activating the  
10 surfaces of the polymeric substrates prior to attaching the acid moiety-containing biomolecule.

### BACKGROUND OF THE INVENTION

15

Hyaluronic acid (HA) is a naturally occurring anionic polysaccharide consisting of alternating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid joined by  $\beta$ -1-3 glucuronic and  $\beta$ -1-4 glucosaminidic bonds, so that the repeating unit is (1-4)- $\beta$ -D-GlcA- (1-3)- $\beta$ -D-GlcNAc. HA is one of many glycosaminoglycans  
20 (GAGs), including heparin, chondroitin sulfate, and keratin, that are widely distributed throughout the body as components of the extracellular matrix (ECM) of connective tissue.

The interactions of HA with HA-binding proteins (known as hyaladherins) are  
25 important in cell adhesion, growth and migration, inflammation, cancer, metastasis, and wound healing. (Knudson, C.B. and Knudson, W., 1993, *Hyaluronan-Binding Proteins in Development, Tissue Homeostasis, and Disease*, FASEB, pp. 1233-1241; Underhill, C.B. in *The Biology of Hyaluronan*, C. Foundation, Editor, 1989; J. Wiley & Sons, Ltd., Chichester, U.K., pp. 87-106; Turley, E.A., in *The Biology of Hyaluronan*, C. Foundation, Ed., 1989, J. Wiley & Sons, Ltd., Chichester, U.K., pp. 121-137; Weigel, P.R., *et al.* in *The Biology of Hyaluronan*, C. Foundation, Ed., 1989, J. Wiley & Sons, Ltd., Chichester, U.K., pp. 248-264). The lack of immunogenicity makes HA an  
30 attractive building block for the design of novel biomaterials. (Drobnik, J. 1994, *Hyaluronan in Drug Delivery*, Adv. Drug Delivery Res. 7:295-308; Swann, D.A. and

J.W. Kuo, in *Biomaterials: Novel Materials from Biological Sources*, Byrom, Ed., 1991, Stockton Press, New York, N.Y. pp. 87-305; Vercruysse, K.P. and G.D. Prestwich, 1998, Hyaluronate Derivatives in Drug Delivery, *Critical Reviews in Therapeutic Drug Carrier Systems* 15:513-555; Prestwich, G. and K. Vercruysse, 1998, 5 Therapeutic Applications of Hyaluronic Acid and Hyaluronan Derivatives, *Pharmaceutical Science & Technology Today* 1(1): 42-43.) Chemically-modified HA has been used in the controlled release of pharmacologically-active compounds, (see Hume, L.R., Lee, H.K., Benedetti, L., Sanzgiri, Y.D., Topp, E.M., and Stella, V.J., 1994, Ocular Sustained Delivery of Prednisolone Using Hyaluronic Acid Benzyl Ester 10 Films, *Int. J. Pharm.* 111:295-298; Sanzgiri, Y.D., Maschi, S., Crescenzi, V., Callegaro, L., Topp, E.M., and Stella V.J., 1993, Gellan-Based Systems for Ophthalmic Sustained Delivery of Methylprednisolone, *J. Controlled Release* 26:195-201) in cell encapsulation matrices, in mammary implants, (see Lin, K., Bartlett, S.P., Matsuo, K., Livolsi, V.A., Parry, C., Hass, B., and Whitaker, L.A., 1994, Hyaluronic Acid-Filled 15 Mammary Implants: an Experimental Study *Plast. Reconstr. Surg.* 94, 306-315) and in materials for wound treatment (Goa, K.L. and Benfield, P., 1994, Hyaluronic Acid - a Review of Its Pharmacology and Use as a Surgical Aid in Ophthalmology, and Its Therapeutic Potential in Joint Disease and Wound Healing, *Drugs* 47: 536-566). Carboxymethylcellulose-HA has been fabricated into bioresorbable membranes, 20 currently approved for sale by Genzyme Corporation as Seprafilm® for prevention of surgical adhesions. Hydrazide derivatives of HA oligosaccharides (Pouyani, T., and Prestwich, G.D., 1994, Functionalized derivatives of Hyaluronic Acid Oligosaccharides - Drug Carriers and Novel Biomaterials, *Bioconj.Chem.* 5:339-347) and native high mass HA (Pouyani, T., Harbison, G.S., and Prestwich, G.D., 1994, Novel Hydrogels of 25 Hyaluronic Acid: Synthesis, Surface Morphology, and Solid-State NMR, *J. Am. Chem. Soc.* 116:7515-7522) have been prepared, yielding hydrosols and hydrogels with covalently-bound ligands. However, materials such as carboxymethylcellulose-HA or a hydrazide-derivatized HA do not satisfy all the requirements for tissue engineering (promotion of cellular adhesion) or prevention of adhesions, so alternative materials 30 must be found. Polymeric surfaces covalently coated with an appropriate biomolecule, such as HA, would provide substrata that would actively enhance attachment and growth of selected cells and prevent attachment/growth of other cells. However,

known methods of surface modification rely on non-covalent adsorption, photo-immobilization, or vigorous chemical activation of the biomolecule.

In view of the foregoing, it will be appreciated that acid-containing  
5 biomolecules covalently coated onto polymeric substrates, and methods of making the same would be significant advancements in the art. The methods disclosed herein involve the use of vigorous conditions to activate the surface of the polymeric substrate, so that subsequent attachment of the acid moiety-containing biomolecule can be performed under mild aqueous conditions.

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#### SUMMARY OF THE INVENTION

It is an object of the present invention to provide novel compositions  
15 comprising activated polymeric substrates having an acid moiety-containing biomolecule covalently bonded via the acid moiety to the substrates. Preferred acid moiety-containing molecules include polysaccharides, oligosaccharides, polynucleotides, oligonucleotides, polypeptides, oligopeptides, peptide nucleic acids, and other backbone-modified oligonucleotide analogs. It is a further object of the  
20 invention to provide methods for making an activated polymeric substrate having an acid moiety-containing biomolecule comprising (a) derivatizing the polymeric substrate to obtain a polymeric substrate having a reactive group on the surface thereof; and (b) covalently bonding an acid moiety-containing biomolecule via the acid moiety to the reactive group. In a further object, the methods involve the addition of spacer groups  
25 onto either or both the biomolecule and the polymeric substrate. It is another object of this invention to provide a cell-supporting material comprising a polymeric substrate having an acid moiety-containing biomolecule covalently bonded via the acid moiety to the substrate wherein cells selectively attach to the acid moiety-containing biomolecule. Such materials enhance attachment and growth of selected cells while preventing or  
30 inhibiting the attachment and growth of other cells.

## DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic representation of "direct coupling" of hyaluronic acid to a polymeric substrate.  $X_1$  can be any of the functionalized derivatives described herein, such as  $-\text{SO}_2\text{NHNH}_2$ , and  $X_2$  can be any covalent linkage described herein between the polymeric substrate and the acid moiety-containing biomolecule, such as  $-\text{NH}-\text{NH}-\text{SO}_2-$ .

Figure 2 shows a schematic representation of "indirect coupling" of a modified hyaluronic acid (HA-ADH) to a polymeric substrate.  $X_1$  can be any of the functionalized derivatives described herein, such as  $-\text{SO}_2\text{NHNH}_2$ , and  $X_2$  can be any covalent linkage described herein between the polymeric substrate and the acid moiety-containing biomolecule, such as  $-\text{NH}-\text{NH}-\text{SO}_2-$ .

## DESCRIPTION OF THE INVENTION

Definitions

In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out herein. As used in this specification and the appended claims:

The terms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

The term "activated polymeric substrate" means a substrate wherein the surface has been modified by the addition of chemically reactive groups.

Detailed Description

Provided herein are compositions of matter comprising an activated polymeric substrate having an acid moiety-containing biomolecule covalently bonded via the acid



moiety to the substrate. As used herein, "via the acid moiety" is a broad term reflecting that the attachment of the biomolecule to the substrate is through the acid moiety on the biomolecule. As described in more detail below, this attachment can be directly to the substrate, or it can be through one or more linker or spacer molecules.

5

The advantages of such biomolecule-coated polymeric substrates are numerous. Firstly, the biomolecule may be modified to include specific bioactive peptides that can mediate cell attachment, for example the cell-adhesion polypeptides such as YIGSR-, SIKVAV- and RGD-containing polypeptides, or other protein or GAG components of the ECM, by chemical modification or adsorption (Vercruysse, K.P. and G.D. Prestwich, 1998, *ibid.*; Prestwich, G.D., D.M. Marecak, J.F. Marecak, K.P. Vercruysse, and M.R. Ziebell, 1998, "Chemical Modification of Hyaluronic Acid for Drug Delivery, Biomaterials and Biochemical Probes", in *The Chemistry, Biology and Medical Applications of Hyaluronan and its Derivatives* (T.C. Laurent, Ed.); Portland Press, London, pp. 43-65). Secondly, the biomolecule, such as HA, is hydrophilic and non-cytotoxic. Thirdly, selected biomolecules can create a lubricious surface which will exhibit minimal adsorption/adhesion of undesired cells, proteins or bacteria. Fourthly, naturally occurring biomolecules such as GAGs, and fragments or analogs thereof, are typically non-immunogenic and non-thrombogenic. Fifthly, the attachment of the biomolecules to a flexible nonbiodegradable surface would provide appropriate mechanical properties for it to function as a support for selective cell growth. Specific applications for a biomolecule-coated polymeric substrate, as disclosed herein, would include support of osteoblast growth for prosthetic hard tissue applications, support of endothelial cell growth for porous or non-porous vascular grafts, support of chondrocyte growth for prosthetic cartilage, or support of hippocampal cells for guided nerve regeneration (Seekel, B.R., et al. 1995, Hyaluronic Acid Through a New Injectable Nerve Guide Delivery System Enhances Peripheral Nerve Regeneration in the Rat, J. Neurosci Res. 40(3):318-324).

30 In preferred embodiments, the activated polymeric substrate has reactive groups selected from the group consisting of amino, carboxyl, carbonyl, sulfonyl, and hydroxyl; and the acid-containing biomolecule can be a polysaccharide,

oligosaccharide, polynucleotide, oligonucleotide, polypeptide, or an oligopeptide. In specific embodiments, these biomolecules can be further modified, such as peptide nucleic acids and other backbone-modified oligonucleotides.

5 In a preferred set of embodiments, the acid moiety-containing biomolecule is a glycosaminoglycan (GAG). Examples of GAGs include hyaluronic acid (HA), sulfated GAG, heparin, chondroitin sulfate, and keratin, which are naturally occurring and widely distributed throughout the body as components of the extracellular matrix (ECM) of connective tissue. The specific chemical structures of these GAGs impart  
10 specific functionality, for example, the viscoelastic properties of HA account for its uniqueness in joint lubrication (see Ghosh, P., 1994, *The Role of Hyaluronic Acid (Hyaluronan) in Health and Disease - Interactions with Cells, Cartilage and Components of Synovial Fluid*, Clin.Exp. Rheumatol 12:75-82). Additional properties of HA that make it suitable as a biomolecule of this invention are described in Band,  
15 P.A., 1998, "Hyaluronan derivatives: chemistry and clinical applications" in *The Chemistry Biology and Medical Applications of Hyaluronan and its Derivatives* (T.C. Laurent, Ed.); Portland Press, London, pp. 33-42; and in Laurent, T.C. and J.R.E. Fraser, 1992, *Hyaluronan* FASEB 6:2397-3404. The size of HA molecules useful in this invention can range from a hexasaccharide (3 repeating disaccharide units) to a  
20 polymer with as many as 30,000 sugar monomers. In a preferred embodiment, the MW of the HA is between 6,000 and 1.2 million daltons.

The general lack of immunogenicity of GAGs and other naturally occurring acid moiety-containing biomolecules makes them attractive components in the compositions  
25 disclosed herein. In a specific embodiment, the use of a GAG polymer as the biomolecule will support and direct nerve growth as well as provide a new tool that allows percutaneous access to the milieu of a regenerating nerve.

In preferred embodiments, the polymeric substrate can be a synthetic polymer,  
30 such as a thermoplastic, thermoset, or an elastomer. In specific embodiments, the thermoplastic is a polyolefin, vinyl, acrylic, fluorocarbon, polyester, polyether, polyamide, or a polyurethane. In other specific embodiments, the thermoset is an



epoxy, phenolic, or a polymer based on aldehydes, ureas, melamines or their derivatives. In other specific embodiments, the elastomer is a diene rubber, silicone rubber, or a polyurethane. In specific embodiments, the polymeric substrate is either polypropylene, polystyrene or polytetrafluoroethylene.

5

In another embodiment, the invention herein described comprises methods of making a derivatized or activated polymeric substrate having an acid moiety-containing biomolecule comprising (a) derivatizing the polymeric substrate so as to place reactive groups on the surface thereof; and (b) covalently bonding an acid moiety-containing biomolecule via the acid moiety to the reactive group. In further embodiments of the methods of this invention, the methods comprise the steps of (a) derivatizing the polymeric substrate so as to place reactive groups on the surface thereof; (b) reacting the reactive group with a reagent having at least two functional groups; and (c) covalently bonding the acid moiety-containing biomolecule via the acid moiety to the polymeric substrate-bound reagent of step (b). In other embodiments of the methods, the reagent with at least two functional groups (i.e. a multifunctional reagent) is first covalently bound to the acid moiety-containing molecule. Then the derivatized biomolecule can be reacted with the activated polymeric substrate. The multifunctional reagent can be any molecule that has at least two chemically reactive groups, which may or may not be the same. In specific embodiments, the multifunctional reagent is selected from the group consisting of di- and poly-carboxylic anhydrides, bisoxiranes, divinyl sulfone, dihydrazides, diamines, polyamines, and polyhydrazides.

In a preferred embodiment of these methods, the surface of the polymeric substrate is activated through plasma processing of the surface of the substrate (see Kaplan, S.L. and P.W. Rose, 1991, Plasma Surface Treatment of Plastics to Enhance Adhesion, Int. J. Adhesion and Adhesives, 11(2):109-113). Plasma-based modification of the polymers is typically performed by placing the substrate in a partially evacuated environment. A power source of radio frequency excites the gas molecules in the chamber, creating a cold plasma that is a mixture of photons, electrons, ions, radicals, and atoms that have the potential to react with the substrate surfaces (Kaplan, S.L., and Rose, P.W., *ibid.*; Pitt, W.G. and Lakenan, J.E., U.S. Patent

No. 5,108,780). Such a mixture actively etches away the polymer surface, leaving polymer fragments containing radicals that can subsequently react with other molecules. Common etching gases are He, Ar, O<sub>2</sub> or CF<sub>4</sub>. Alternatively, one can plasma process the surface using a more reactive gas that both etches and leaves behind various functional groups on the surface. Gases like N<sub>2</sub>, NH<sub>3</sub>, SO<sub>2</sub>, O<sub>2</sub>, CO<sub>2</sub> and H<sub>2</sub>O are commonly used to derivatize the polymer surface with amino, sulfonyl, carboxyl, carbonyl, and hydroxyl species that can interact directly with biomolecules (see Chu, T.J., Caldwell, K.D., Weiss, R.B., Gesteland, R.F., and Pitt, W.G., 1992, Low Fluorescence Background Electrophoresis 13:105-114; Golander, C.G. and Pitt, W.G. 1989, Characterization of Hydrophobicity Gradients Prepared by Means of Radio Frequency Plasma Discharge, Biomaterials 11:32-35) or can be used in further surface derivatization schemes (Nay, J.C., Pitt, W.G., and Armstrong-Carroll, E., 1995, Improving Adhesion in Interleaf Composites Using Plasma Processing, J. Appl. Polym. Sci. 56:461-469; Pitt, W.G., 1989, Fabrication of a Continuous Wettability Gradient by Radio Frequency Plasma Discharge, J. Colloid Interface Sci. 133:223-227; Pitt, W.G., Lakenan, J.L., and Strong, A.B., 1993, The Influence of Plasma Gas Species on the Adhesion of Thermoplastic to Organic Fibers, J. Appl. Polym. Sci. 48:845-856). In a specific embodiment, the method comprises activation of a polymer surface with ammonia plasma followed by succinic anhydride coupling of HA-ADH to the reactive primary amino groups on the polymer surface.

In another embodiment, cell supporting material comprising a polymeric substrate having an acid moiety-containing biomolecule covalently bonded via the acid moiety to the substrate wherein cells selectively attach to the acid moiety-containing biomolecule is provided. The cell-supporting material is ideal for cell attachment and growth, because it (i) has the cell attachment moieties (e.g. polypeptides, receptor ligands, or other ECM components) on the surface of the material or it selectively adsorbs these moieties; (ii) is non-toxic to the cells; (iii) is passive or does not adsorb other proteins, cells, or bacteria; (iv) is non-immunogenic and non-thrombogenic, and (v) has the mechanical properties to function as a support for cell growth. Acid moiety-containing biomolecules, which may or may not be modified with cell-specific

attachment moieties, are covalently bound through the acid moiety, directly or through spacer groups, to the polymeric support which provides the mechanical support for these embodiments.

5           In specific embodiments, the cell attachment moieties are chosen to allow for the selective attachment of certain cell types. For example, an RGD-containing peptide can be covalently bound to the acid moiety of HA. The resulting HA-RGD is then attached to the polymeric substrate, and this composition can be used to selectively recruit endothelial cells, chondrocytes, and platelets. In another embodiment, the  
10       attachment of an REDV-HA biomolecule to the polymeric substrate would result in a composition that selectively recruits only endothelial cells. In another embodiment, the attachment of an IKVAV-HA biomolecule would recruit only neural cells.

          In additional embodiments, HA-coated non-degradable polymers (e.g. PET,  
15       Teflon, PE, hydrogels, etc.) are used to support osteoblast growth for prosthetic hard tissue applications, to support endothelial cell growth for porous or non-porous vascular grafts, to support chondrocyte growth for prosthetic cartilage, and to support hippocampal cells for guided nerve regeneration. In another set of embodiments, HA biomolecules coated onto degradable polymers are used as scaffolding in hard and soft  
20       tissue regeneration, or as short term implants (sutures, bone screws, etc.). In these embodiments, the cells of the desired tissue are either seeded onto the HA-coated polymer prior to implantation, or the HA-coated material is first implanted, with the targeted cells being recruited through attachment to the specific adhesion molecules on the modified HA. In these embodiments, less desirable proteins and cells are excluded  
25       from attachment to the implant due to the nonadhesive nature of the HA coated polymeric substrate.

## EXAMPLES

Example 1. Surface modification Via Plasma Processing.

5 Radio frequency plasma processing, (as described in Golander, C.G. and Pitt, W.G., 1990 Biomaterials 11:32-35), was used to chemically derivatize polypropylene (PP), polystyrene (PS), and Teflon(poly(TFE)) surfaces. PP sheets were obtained from Exxon (Pottsville, PA), PS samples were obtained from sterile petri dishes (Fisher Scientific, cat. no. 08-757-14), and PTFE sheets were purchased from Ain Plastics  
10 (Grove City, OH). PP, PS, and PTFE were cut into 10 x 10-cm coupons and cleaned by immersion in EtOH overnight.

The polymer coupons were plasma processed using argon, ammonia or a combination of the two by centering the coupons between parallel metal plates, 20 cm  
15 from each plate, in a Plasma Science 0500 reactor and evacuating the chamber to the base pressure. The chamber was then filled with argon or ammonia and raised to the processing pressure. Once the processing pressure was reached, the RF generator was activated at the desired wattage for the set process time. The argon-treated coupons were exposed to argon plasma for one minute at a throttle pressure of 0.500 Torr and a  
20 gas flow rate of 0.0100 standard liters per minute. The ammonia-treated coupons were treated for 2 minutes at a throttle pressure of 0.375 Torr and a gas flow rate of 0.0552 standard liters per minute. For the combination treatment, polymer coupons were first processed in a 13.56 MHz Ar plasma, then the chamber was evacuated to base pressure, and the coupons were exposed to an NH<sub>3</sub> plasma process, followed by soaking in H<sub>2</sub> to  
25 derivatize /activate the surface with primary amino groups for subsequent reaction via carbodiimide to couple HA to the surface. The N concentration on the surface was measured as a function of RF power (50 to 300 W), gas pressure (0.1 to 0.6 Torr), gas flow rate (100 to 600 pmole/s), and exposure time (0.5 to 5 min). Activation with primary amino groups is preferred for the PS and PP surfaces. For PTFE, the preferred  
30 activation is through the use of an SO<sub>2</sub> or CO<sub>2</sub> plasma to introduce sulfonyl or carbonyl functionalities that could subsequently be reacted with hydrazine to produce a sulfonyl hydrazide or hydrazone, which could then undergo the carbodiimide-mediated

condensation with HA or a diacid spacer reagent.

In one experiment, five materials were produced: (1) unmodified polypropylene (PP) film, (2) argon-plasma etched PP, (3) ammonia-plasma etched PP, (4) argon-then ammonia-plasma etched PP, and (5) argon-then ammonia-plasma etched PP, followed by an ammonia quenching (capping) step. These samples and azlactone modified polyethylene (PE) films can also be used as substrates for HA coating. Other substrate modifications include the ammonia plasma treatment of polystyrene (PS) surfaces and sulfonyl plasma activation of PP, PS, or Teflon surfaces.

10

#### Example 2. Modification of HA.

Modification of HA via carbodiimide-mediated coupling of carboxylic acid hydrazides to the glucuronic acid carboxylates of HA has been described (Pouyani and Prestwich, *supra*, at 339-347; Pouyani, Harbison, and Prestwich, *supra*, at 7515-7522.) Alternatively, sulfonyl hydrazides (e.g., toluene sulfonyl hydrazide and Texas Red sulfonyl hydrazide) can also be conjugated to HA carboxylic acid groups with excellent efficiency. Three size ranges of native HA, ranging from a MW of 700,000-1,200,000 daltons (available from Clear Solutions Biotech, Inc. New York) were used. Alternatively, oligosaccharides and polysaccharides containing at least 3 repeating disaccharide units of HA can be used.

20

#### (A) Direct coupling

25

This procedure is diagrammed in Figure 1. A hydrazide-activated surface was treated with each of the three sizes of native HA at a concentration of 6 mg/ml in pH 4.75 buffer using N-ethyl, N-(3-dimethylaminopropyl)carbodiimide (EDCI), i.e. the  $RN=C=NR'$  of Figure 1, as the coupling agent. This method is preferred for oligosaccharides of HA and smaller polysaccharide classes of HA, since the HA carboxylates will be sufficiently "close" to the activated polymeric surface.

30



(B) Indirect coupling

This procedure is diagrammed in Figure 2. The use of a simple dicarboxylic acid, e.g., adipic, succinic or glutaric acid, or a polymeric acid such as polyethylene glycol (PEG) diacid, can extend the reactive group of the activated substrate or of the acid moiety-containing biomolecule further from the substrate surface or biomolecule backbone, respectively. The chemical functional group on the surface may also permit use of an anhydride, such as succinic or glutaric anhydride, or an activated ester form of the dicarboxylic acid for more efficient coupling chemistry. Figure 2 shows an example in which both the polymeric substrate and the acid-moiety containing biomolecule have been extended. EDCI-mediated coupling of the activated dicarboxylic acid-derivatized polymeric surface to adipic dihydrazide (ADH)-modified HA then covalently attaches the HA to the surface.

15 Example 3. Chemical characterization of modified surfaces.

Plasma-modified surfaces were characterized using standard contact angle and ESCA techniques to establish the type and amount of derivatization. SEM and AFM were used to monitor any change in surface roughness.

20

Example 4. HA Conjugation to Activated Polymeric Substrates and Surface Analysis

25 Samples (15 mm x 20 mm) of each PP film type produced in Example 1 were treated to achieve HA attachment. Optimization of these treatments can be monitored through detection of a significant change in the water contact angle of the coated surface. HA was furnished by Clear Solutions Biotechnology, Inc. (Stony Brook, NY) and adipic dihydrazide modified HA (HA-ADH) was prepared as previously described (Pouyani, T. and Prestwich, G.D., 1994, *ibid.*) with modifications (Vercruysse, K.P., 30 Marecak, D.M., Marecak, J.R., and Prestwich, G.D., 1997, Synthesis and *in vitro* Degradation of New Polyvalent Hydrazide Cross-linked Hydrogels of Hyaluronic Acid,



Bioconjugate Chemistry 8(5):686-694). Bis-Tris HCl was obtained from Sigma (St.Louis, MO).

For a negative control, a sample was placed in a beaker containing 2 mg/ml HA  
5 in 50 mM bis-Tris-HCl solution overnight. For attaching HA, a solution of 1.2 mg/ml  
HA, 50 mM bis-Tris HCl and 1 mM 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide  
HCl (EDCI; coupling reagent) was added and incubated overnight. Alternatively,  
aminated surfaces of the polymeric substrate were first converted to carboxylic acid-  
modified surfaces by incubation with a solution of 10 mM succinic anhydride in dry  
10 DMF (for PP and PTFE) or in EtOH solution (for PS) for 10 h, followed by washing for  
30 minutes in 10 mls distilled water. The succinate-modified surfaces were then  
incubated overnight with HA-ADH in 50 mM bis-Tris HCl and 1 mM EDCI. The pH  
of all bis-Tris solutions was 4-4.75. All reactions were performed in an orbital shaker  
at 90 rpm and 6 °C to minimize bacterial growth. The samples were then washed  
15 overnight in doubly distilled water at a low flow rate in order to remove any physically  
adsorbed HA.

Treated and untreated surfaces were evaluated using FTIR spectroscopy, XPS,  
and water contact angle measurements. FTIR spectra were obtained on a Nicolet 730  
20 FTIR spectrometer using a 45° Ge crystal and a SpectraTek variable angle ATR  
apparatus. Contact angles were measured using a Rame-Hart argonometer. XPS  
analysis was performed on a Fisons multi technique ESCALAB 2201-XL surface  
analysis instrument. The XPS survey spectra and high resolution spectra were collected  
using Al K $\alpha$  X-rays at constant analyzer energies of 100 and 20 eV respectively. The  
25 FTIR spectra gave the most conclusive results for PP and PTFE, but is less informative  
for PS analysis, due to the poor contact between the rigid PS samples and the Ge  
crystal. All polymers treated with ammonia, argon or argon-ammonia plasma had  
water contact angles that were significantly less ( $p < .01$ ) than the clean untreated  
sample. Reduction of contact angle is consistent with the oxidation and/or amination of  
30 the hydrophobic polymers. The lyophilized product may show a visible fibrous layer of  
HA on the film. Testing for water contact angle, whether or not a fibrous layer is  
visible, and finding complete spreading of the droplet, indicates attachment with HA.

The XPS analysis shows an increase in oxygen and nitrogen following plasma treatment. FTIR spectra were collected on treated and untreated PP films. The PP has no absorbance at  $1045\text{ cm}^{-1}$  (ether) or  $1643\text{ cm}^{-1}$  (carbonyl) and the HA has very little absorbance at  $1458\text{ cm}^{-1}$  (CH bending). These spectra show no change in the PP spectra after ammonia plasma treatment. There is a trace of HA adsorbed on the non-plasma treated PP following the derivatization procedure described herein. This is expected, as non-covalent adsorption of high mass HA to plastic surfaces is commonly observed. However, when the PP was plasma activated with argon, ammonia, or both, the spectra of pure HA is superimposed onto the PP spectrum indicating significant attachment. A semi-quantitative analysis of the amount of HA attachment was performed by taking the ratio of the  $1045\text{ cm}^{-1}$  and  $1643\text{ cm}^{-1}$  peaks to the  $1458\text{ cm}^{-1}$  peak. These ratios are an indication of the relative amount of HA bound on each sample. The ratios for the non-plasma treated, argon, ammonia, and argon/ammonia treated samples are 0.12, 1.39, 2.57, and 1.27, respectively.

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#### Example 5. Cell attachment/growth

Studies of cell growth on modified surfaces use PC12 and HL60 cells. Cell adhesion, morphological changes, and spreading are typically monitored during a 2-week culture period. Surfaces modified with specific adhesins, such as RGD, REDV, IKVAV, or YIGSR peptides, were used to promote cell adhesion and spreading; for these studies, chondrocytes, platelets, epithelial cells, and neurites were employed.

25

It is to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

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## CLAIMS

We claim:

1. A composition of matter comprising an activated polymeric substrate having an acid moiety-containing biomolecule covalently bonded via the acid moiety to the substrate.
2. The composition of claim 1 wherein the activated polymeric substrate has reactive groups selected from the group consisting of amino, carboxyl, carbonyl, sulfonyl, and hydroxyl.
3. The composition of claim 1 wherein the acid-containing biomolecule is selected from the group consisting of polysaccharides, oligosaccharides, polynucleotides, oligonucleotides, polypeptides, oligopeptides, peptide nucleic acids, and backbone-modified oligonucleotide analogs.
4. The composition of claim 3 wherein the acid moiety-containing biomolecule is a glycosaminoglycan.
5. The composition of claim 4 wherein the glycosaminoglycan is selected from the group consisting of hyaluronic acid, heparin, chondroitin sulfate, and keratin.
6. The composition of claim 1 wherein the polymeric substrate is a synthetic polymer.
7. The composition of claim 6 wherein the synthetic polymer is selected from the group consisting of thermoplastics, thermosets, and elastomers.
8. The composition of claim 7 wherein the thermoplastic is selected from the group consisting of polyolefins, vinyls, acrylics, fluorocarbons, polyesters, polyethers, polyamides, and polyurethanes.

9. The composition of claim 7 wherein the thermoset is selected from the group consisting of epoxies, phenolics, or polymers based on aldehydes, ureas, melamines or their derivatives.
10. The composition of claim 7 wherein the elastomer is selected from the group consisting of diene rubbers, silicone rubbers, and polyurethanes.
11. The composition of claim 1 wherein the polymeric substrate is selected from the group consisting of polypropylene, polystyrene and polytetrafluoroethylene.
12. A method of making an activated polymeric substrate having an acid moiety-containing biomolecule comprising:
  - (a) derivatizing the polymeric substrate so as to place reactive groups on the surface thereof; and
  - (b) covalently bonding an acid moiety-containing biomolecule via the acid moiety to the reactive group.
13. A method of making an activated polymeric substrate having an acid moiety-containing biomolecule comprising:
  - (a) derivatizing the polymeric substrate so as to place reactive groups on the surface thereof;
  - (b) reacting the reactive group with a reagent having at least two functional groups; and
  - (c) covalently bonding the acid moiety-containing biomolecule via the acid moiety to the polymeric substrate-bound reagent of step (b).
14. The method of claim 12 wherein step (a) is accomplished through plasma

processing of the polymeric substrate.

15. The method of claim 13 wherein step (a) is accomplished through plasma processing of the polymeric substrate.
16. The method of claim 12 wherein the acid moiety-containing biomolecule is modified by the addition of spacer groups at the acid moiety.
17. The method of claim 13 wherein the acid moiety-containing biomolecule is modified by the addition of spacer groups at the acid moiety.
18. The method of claim 13 wherein the reagent with at least two functional groups is selected from the group consisting of di- and poly-carboxylic anhydrides, bisoxiranes, divinyl sulfone, dihydrazides, diamines, polyhydrazides and polyamines.
19. A cell-supporting material comprising a polymeric substrate having an acid moiety-containing biomolecule covalently bonded via the acid moiety to the substrate wherein cells selectively attach to the acid moiety-containing biomolecule.
20. The material of claim 19 wherein the material is an implant for use in animals, including humans.
21. The material of claim 20 wherein the cells are attached prior to implantation of the material.
22. The material of claim 20 wherein the cells attach after implantation of the biomaterial.

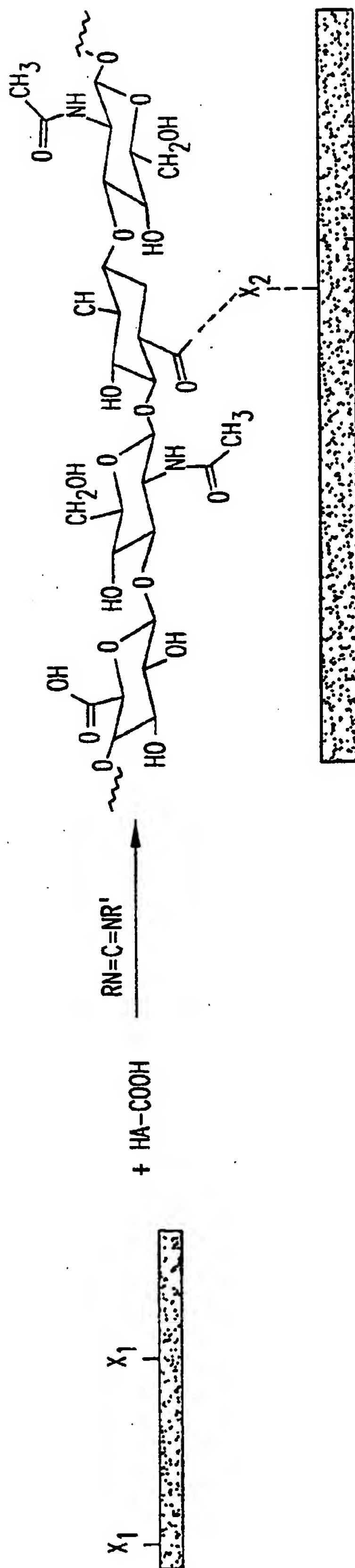


FIG.1



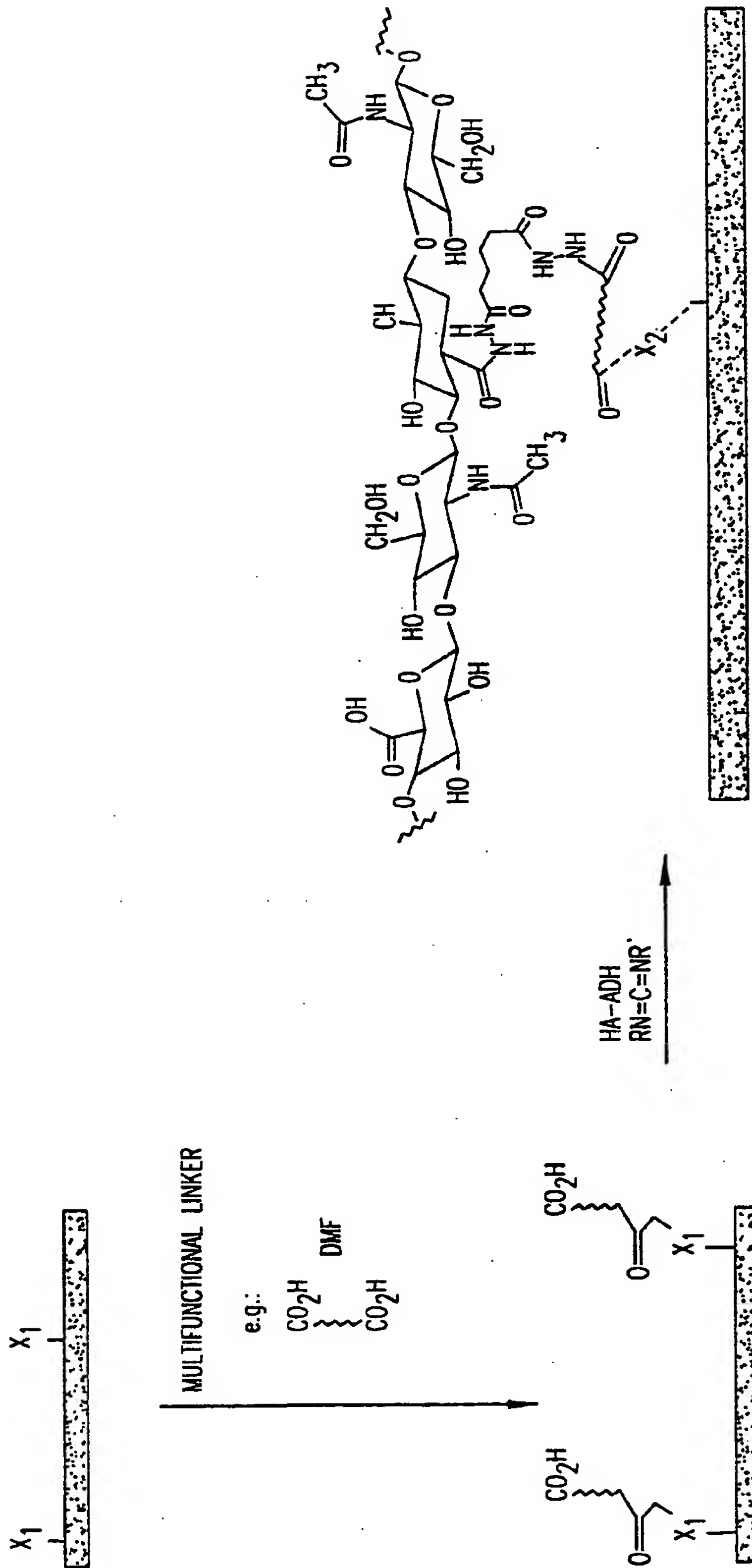


FIG.2

## INTERNATIONAL SEARCH REPORT

Internatio Application No

PCT/US 00/01028

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61L33/00 A61L31/16 A61L31/10 A61L31/04

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 608 095 A (MEDTRONIC INC) 27 July 1994 (1994-07-27) page 2, line 35 -page 3, line 9 page 3, line 17 - line 32 page 3, line 41 -page 4, line 8 ----	1-13, 19-22
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☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&amp;" document member of the same patent family

Date of the actual completion of the International search

11 May 2000

Date of mailing of the International search report

13/06/2000

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	<p>EP 0 367 489 A (BAXTER INT) 9 May 1990 (1990-05-09)</p> <p>page 4, line 52 - line 54 page 5, line 9 - line 42</p>	<p>1-3, 6-13, 16-18</p>
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Information on patent family members

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